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Nondeletional α-Thalassemia: First Description of $\alpha^{Hph}\alpha$ and $\alpha^{Nco}\alpha$ Mutations in a Spanish Population

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Several different deletions underlie the molecular basis of α-thalassemia. The most common α-thalassemia determinant in Spain is the rightward deletion ($-\alpha^{3.7}$). To our knowledge, however, no cases of α-thalassemia due to nondeletional mutations have so far been described in this particular Mediterranean area. Here, we report the existence of nondeletional forms of α-thalassemia in ten Spanish families. The α_2-globin gene was characterized in ten unrelated patients and their relatives only when the presence of deletional α-thalassemia was ruled out. The α_2-globin gene analysis was performed using the polymerase chain reaction (PCR) followed by restriction enzyme analysis or by allele-specific priming. This allowed the identification of a 5-base pair (bp) deletion at the donor site of IVS I ($\alpha^{Hph}\alpha$) in 9 cases and the α_2 initiation codon mutation ($\alpha^{Nco}\alpha$) in one case. Although these α_2-globin gene mutations are found in other Mediterranean areas, our results demonstrate their presence in the Spanish population and suggest that the $\alpha^{Hph}\alpha/\alpha\alpha$ genotype is probably the most common nondeletional form of α-thalassemia in Spain. © 1996 Wiley-Liss, Inc.

Key words: α-thalassemia, nondeletional mutations, PCR, restriction enzyme analysis, Spain

INTRODUCTION

The α-thalassemia (α-thal) syndromes are relatively frequent genetic disorders caused by the absence (α^0-thal or α-thal-1) or a decrease (α^+-thal or α-thal-2) in the synthesis of globin α-chains [1,2]. The human α-globin genes are clustered on the short arm of chromosome 16 and arranged in the order: $5'$-ζ_2-$\psi\zeta_1$-$\psi\alpha_2$-$\psi\alpha_1$-α_2-α_1-θ_1-$3'$ [3,4]. The different clinical phenotypes of α-thal syndromes result from molecular defects located within this α gene complex. Usually, they are due to large deletions that remove either one ($-\alpha$) or both ($--$) α globin genes (α_2 and α_1) from one chromosome whose size and position differs according to population. Less frequently, they result from nondeletional mutations involving one or a few nucleotides most commonly within the α_2-gene ($\alpha^T\alpha$) or occasionally in the α_1-gene ($\alpha\alpha^T$) [5].

The predominant α-thal-2 defect is the rightward deletion ($-\alpha^{3.7}$), which is prevalent in people from most tropical and subtropical geographical areas, including Africans, the black population of America, and people from the Mediterranean, Southeast Asia, and some Pacific Islands [5]. The less common leftward deletion ($-\alpha^{4.2}$) is a polymorphic α-thal-2 mainly described in Southeast Asia and the Middle East [6]. The most frequent α-thal-1 determinants are the $--^{MED}$ and $-(\alpha)^{20.5}$ deletions in the Mediterranean area [7] and the $--^{SEA}$ deletion in Southeast Asia [8]. Like other α^0-thal deletions, all these determinants have been reported in subjects of Spanish origin [9–12].

The molecular basis of nondeletional α-thal determinants is well known and, with the exception of three cases, due to mutations in the α_1-gene [13–15], all mutations involve the dominant α_2-globin gene [5]. Four of these mutations are predominant in the Mediterranean area: (1) a deletion of 5 bp (GGTGAGGCT→GGCT) involving the $5'$ splice junction of IVS I ($\alpha^{Hph}\alpha$) [16], (2) a mutation that changes the initiation codon ATG to ACG ($\alpha^{Nco}\alpha$) [17], (3) a termination codon mutation (α142

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TAA→AAA) associated with Hb Icaria ($\alpha^{Ic}\alpha$) [18], and (4) a polyadenylation site mutation ($\alpha^{TSaudi}\alpha$) due to a single base mutation (AATAAA→AATAAG) in the poly A signal of the α_2-globin gene [19]. Recently, two new polyadenylation site mutations have been described: the ATTAAA→AATGAA mutation in a Turkish family [20] and the two-base deletion (AATAAA→AATA) in an Indian patient [21].

In this study, DNA restriction analysis with Southern Blot was performed in a large group of Spanish subjects with family microcytosis. Ten patients were suspected of being carriers of nondeletional α-thalassemia. Our aim was to identify the molecular defect in these cases, by selective amplification of the α_2-globin gene using the polymerase chain reaction (PCR), and by testing for the four most frequent nondeletion α-thalassemia mutations previously described in the Mediterranean area, but to our knowledge not yet reported in Spain.

MATERIAL AND METHODS

Subjects

A total of 21 subjects from 10 unreleated Spanish families with microcytic red blood cells not due to iron deficiency and with normal HbA_2 and HbFetal (HbF) were included in this study. These cases were selected from a group of patients sent to the Hospital Clínic i Provincial de Barcelona for diagnosis of α-thalassemia over the last 3 years. Whole blood samples were collected using EDTA-K_3 as anticoagulant and routine hematological data were obtained with an H*2 (Technicon) hematological analyzer. Hemoglobin concentration HbA_2 and HbF measurements were carried out by standard methods [22].

DNA Restriction Analysis

Genomic DNA was isolated from peripheral blood leukocytes [23] from normal and affected individuals and analyzed by Southern Blot hybridation [24]. Briefly, 10 μg of genomic DNA was digested with the restriction endonucleases *Bgl*II, *Bam*HI, and *Xba*I according to the manufacturer's specifications. The DNA fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with the following probes: 1.5 kb *Pst*I α, 1.8 kb *Sac*I ζ, and a ψα-gene-specific probe [11], labeled by random primers with α-^{32}P-dCTP [25].

Selective Amplification of the α_2-Globin Gene

The entire α_2-globin gene from each patient and normal individual was selectively amplified by PCR [26] using specific previously described primers (C8 5′-GAGCCTGGCCAAACCATCAC-3 and C3 5′-CCATTGTTGGCACATTCCGG-3) [27,28] producing a 1,943-bp fragment. PCR was performed with a DNA thermal cycler (Perkin-Elmer Cetus Instruments). The reaction mixture (100 μl) contained 50–100 ng genomic DNA, 40 pmol of each primer with 16.6 mM $(NH_4)_2$ SO_4, 67 mM Tris–HCl at pH 8.8, 2.5 mM $MgCl_2$, 67 μM Na_2 EDTA, 160 μg bovine serum albumin (BSA) per ml, 10 mM β-mercaptoethanol, 10% dimethyl sulfoxide (DMSO), 0.2 mM dNTPs, and 2 U of Taq polymerase (GIBCO-BRL, Gaithersburg, MD USA). After 10 min at 94°C, a total of 30 cycles were run under the following conditions: denaturation at 94°C for 45 sec, annealing at 56°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min in the last cycle. Following amplification, 10 μl of the product was analyzed by 1% agarose gel electrophoresis at 100 V for 45 min, stained with ethidium bromide, and visualized on an ultraviolet (UV) transilluminator.

TABLE I. Detection of Nondeletional α-Thalassemia Mutations by Restriction Enzyme Analysis of a Specific α_2 PCR Product (1,943 bp)

	Haplotype			
ER	$\alpha^{Hph}\alpha$	$\alpha^{Nco}\alpha$	$\alpha^{Ic}\alpha$	αα
*Hph*I	1395, 236, 163, 97, 32, 15			1078, 322, 236, 163, 97, 32, 15
*Nco*I		1943		1094, 849
*Mse*I			1379, 564	1379, 401, 163

ER, restriction enzyme.

PCR-Restriction Endonuclease Analysis

To test for the presence of three nondeletional α-thal mutations, 25 μl of the amplified α_2-globin gene fragment was digested according to the manufacturer's instructions alongside positive and negative controls with the appropriate restriction enzyme as follows: *Mse*I for the termination codon mutation in the α_2-globin gene ($\alpha^{Ic}\alpha$), *Hph*I for the deletion of 5 bp at the donor site of IVS I in the α_2-globin gene ($\alpha^{Hph}\alpha$), and *Nco*I for the α_2 initiation codon mutation ($\alpha^{Nco}\alpha$). The digested DNA fragments were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The expected fragment sizes (bp) of the restricted PCR product for each specific mutation are listed in Table I.

Allele-Specific Priming

The α_2 polyadenylation site mutation ($\alpha^{TSaudi}\alpha$) detection was carried out as previously described [27]. An allele-specific primer, SPA (5′-GCTGCCGCCCACTCACACC-3′) was used in combination with C8 in a second PCR using 1 μl of 1:1,000 dilution of the selectively amplified α_2-globin gene. As a control a third primer, C10 (5′-ACGGTTGAGGGTGGCCTGT-3′) was included in the reaction mixture (25 μl) amplifying a fragment of 1,261 bp together with primer C8. A total of 20 cycles

TABLE II. Hematological Data and Haplotypes from 10 Spanish Patients With α-Thalassemia

	Propositus									
	1	2	3	4	5	6	7	8	9	10
Sex	F	F	M	M	M	M	M	F	M	F
Age (yr)	14	5	28	20	18	15	74	12	20	31
RBC ($\times 10^{12}$/L)	5.1	5.1	6.3	5.5	5.8	5.3	4.9	5.2	6.1	4.9
Hb (g/L)	130	110	150	141	147	128	110	124	151	132
MCH (pG)	25.4	21.6	23.6	25.7	25.3	24.4	22.2	23.6	24.9	26.7
MCHC (g/L)	326	318	322	321	319	307	293	323	326	342
MCV (fL)	74.6	67.9	72.7	70.2	79.6	79.2	75.8	73.2	76.3	78.2
Retis (%)	0.6	0.8	0.9	0.9	0.7	0.6	0.8	0.7	0.5	0.7
HbA_2 (%)[a]	2.9	2.9	2.6	2.5	2.6	2.5	2.5	2.2	2.3	2.5
HbF (%)[b]	0.1	0.4	0.3	0.2	0.2	0.1	0.1	0.6	1.0	0.5
Ferritin (μg/L)	23	36	154	103	65	210	240	43	180	39
Haplotype	$\alpha^{Nco}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$	$\alpha^{Hph}\alpha$

M, male; F, female; ND, not determined.
[a]Normal values of HbA_2 (%): 2.4–3.2.
[b]Normal values of HbF (%): 0.2–2.

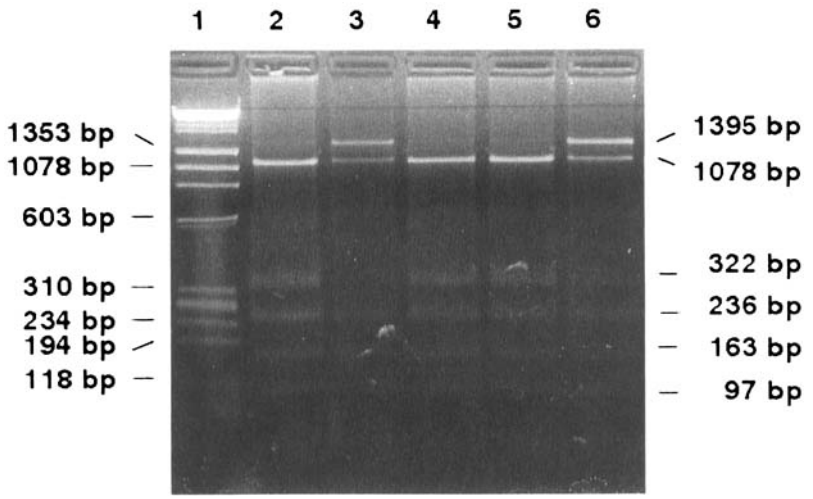

Fig. 1. Restriction enzyme analysis of the 5-bp deletion at the donor site of IVS-1 in the α_2-globin gene. Since a *Hph*I site is destroyed by this mutation, the 1,943-bp amplified fragment, including the α_2-globin gene of the mutant samples, is cleaved to 1,395-, 1,078-, 322-, 236-, 163-, 97-, 32-, and 15-bp fragments, as opposed to 1,078-, 322-, 236-, 163-, 97-, 32-, and 15-bp in the wild type. Molecular-weight markers: λ phage cut with *Hin*dIII and ϕX 174 DNA cut with *Hae*III run together (lane 1), normal control (lane 2), patients with $\alpha^{Hph}\alpha/\alpha\alpha$ genotype (lanes 3, 6), patients without this mutation (lanes 4, 5). The 32- and 15-bp fragments can not be distinguished in the 2% agarose gel used.

were run under the following conditions: denaturation at 95°C for 10 min in the first cycle and 1 min in each subsequent cycle, annealing for 30 sec at 53°C and extension at 72°C for 3 min (7 min in the last cycle).

RESULTS

The results of hematological studies and Hb measurements (HbA_2 and HbF) in the probands are summarized in Table II. The samples were first tested for the most common deletions that lead to α-thal phenotype by DNA restriction mapping after digestion of genomic DNA with *Bam*HI and *Bgl*II and hybridization with the α-specific probe. In all cases, a fragment of 14 Kb with *Bam*HI, and two fragments of 12 and 7 kb with *Bgl*II, characteristic of normal genotype, were obtained. Furthermore, analysis of *Bgl*II using the ζ probe provided two normal restriction fragments of 12 and 11 kb, and digestion with *Xba*I followed by hybridization with $--^{SPAN}$ deletion-specific ψα-gene probe [11] showed no band abnormalities (data not shown).

The normal Southern Blot pattern obtained in all patients suggested that they might be carriers of nondeletional forms of α-thal. For this reason, all these cases

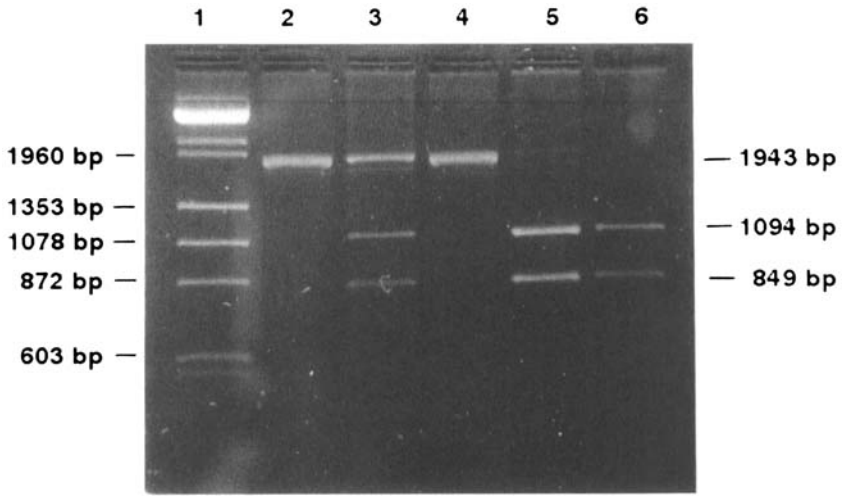

Fig. 2. *Nco*I cleavage of the DNA fragment containing the α_2 initiation codon mutation. The 1943-bp amplified fragment including the α_2-globin gene of the heterozygote patient for this mutation is digested to 1943-, 1094-, and 849-bp fragments, as opposed to 1,094- and 849-bp in the normal control. Molecular-weight markers: λ phage cut with *Hin*dIII and ϕX 174 DNA cut with *Hae*III run together (lane 1), amplified undigested fragments from the patient and normal control (lanes 2, 4), patient with $\alpha^{Nco}\alpha/\alpha\alpha$ genotype (lane 3), digested fragments from normal controls (lanes 5, 6).

were tested for the nondeletional α-thal mutations most frequently encountered in other Mediterranean regions.

A selective fragment of 1,943 bp spanning the α_2-globin gene was amplified by PCR in all cases. All fragments obtained were analyzed as described under Materials and Methods. In nine unrelated cases, the cleavage with *Hph*I produced a 1,395-bp abnormal fragment that is not present in negative controls (Fig. 1). This restriction pattern is characteristic of the heterozygous state for the 5-bp deletion at the donor site of IVS I in the α_2-globin gene ($\alpha\alpha/\alpha^{Hph}\alpha$); thus, this mutation abolishes one of the restriction sites of the *Hph*I endonuclease. Family studies demonstrated the genotype $\alpha\alpha/\alpha^{Hph}\alpha$ in six relatives.

One case was identified as heterozygous for the α_2 initiation codon mutation ($\alpha\alpha/\alpha^{Nco}\alpha$) because the digestion with *Nco*I produced a 1,943-bp abnormal fragment, indicating that this specific mutation abolishes a restriction site for the *Nco*I endonuclease (Fig. 2). The type and frequency of the different nondeletional genotypes found in the patients and their relatives (a total of 21 cases) are summarized in Table III. Screening by allele-specific priming in order to identify a α_2 polyadenylation site mutation ($\alpha^{TSaudi}\alpha$) was unsuccessful (Fig. 3). Likewise, the digestion of the PCR product with *Mse*I demonstrated the absence of the termination codon mutation in the α_2-globin gene ($\alpha^{Ic}\alpha$).

TABLE III. Summary of Genotypes Observed in 10 Spanish Patients With Nondeletional α-Thalassemia and Their Relatives*

Mutation	Genotype	Cases	Frequency (%)
5-bp deletion at IVSI	$\alpha^{Hph}\alpha/\alpha\alpha$	15	71
Poly A signal	$\alpha^{TSaudi}\alpha/\alpha\alpha$	0	0
Initiation codon	$\alpha^{Nco}\alpha/\alpha\alpha$	1	5
Termination codon (Hb Icaria)	$\alpha^{Ic}\alpha/\alpha\alpha$	0	0
Not present[a]	$\alpha\alpha/\alpha\alpha$	4	24

*All identified mutations are located in the α_2-globin gene.
[a]Relatives in which the mutation was not present.

DISCUSSION

Recent studies of direct gene analysis [29] have demonstrated that the frequency of α-thal in Spain is estimated to be 0.0125, where the 3.7 kb ($-\alpha^{3.7}$) deletion is the most frequent variant of the α-thal-2 defect. Furthermore, different α^0 genotypes have been described in a minority of cases in Spain [9,11] although the incidence of nondeletional α-thal mutations is unknown.

Southern blot analysis can identify all major α-thal gene rearrangements, but not small deletions or point mutations (usually affecting the α_2-gene). For this reason, the present study used selective amplification of the α_2-gene, in order to further investigate the molecular defect in ten unrelated Spanish patients with the α-thal trait. In nine cases, PCR-RE revealed a pentanucleotide deletion at the 5′ donor site of IVS I of the α_2-gene. This deletion of T-G-A-G-G, following the G of the invariant GT dinucleotide normally located within the splice junction, alters RNA processing and therefore abolishes the RNA splicing from the normal donor site, introducing a new donor consensus within exon I of the α_2-gene [4]. Family studies led to the identification of six additional individuals with the $\alpha\alpha/\alpha^{Hph}\alpha$ genotype. This mutation was originally

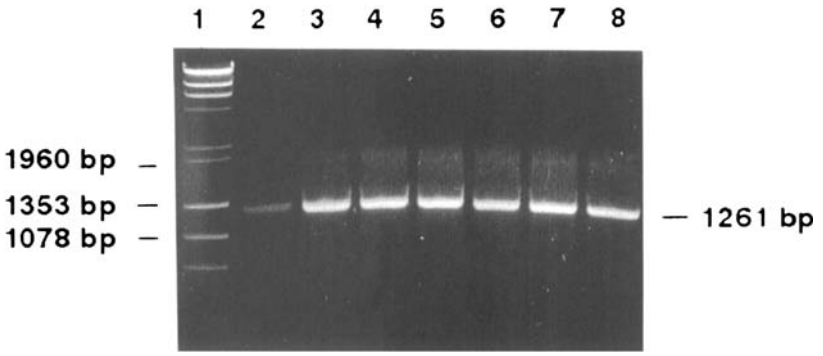

Fig. 3. PCR analysis of the α_2 polyadenylation site mutation ($\alpha^{TSaudi}\alpha$) by allele-specific priming. In all cases, an internal PCR control band of 1,261 bp is present. In all patients studied, the expected 1,897-bp band was not present, thus indicating the absence of this mutation. Molecular-weight markers: λ phage cut with *Hin*dIII and ϕX 174 DNA cut with *Hae*III run together (lane 1), normal control (lanes 2, 3), patients without this mutation

found in Italy [16] and later reported in Turkey and Greece [30]. The fact that our patients came from different geographical areas of the Iberian peninsula (Barcelona, Valencia, Avila) as well as from Canary Islands (Tenerife) and Menorca gives further support to the supposition that the distribution of this nondeletional α-thal mutation within the Mediterranean region is widespread.

In one proband from Barcelona, we have identified the heterozygous condition of the $\alpha\alpha/\alpha^{Nco}\alpha$ initiation codon mutation due to the change of ATG to ACG in the α_2-gene, which abolishes the gene function by altering the mRNA translation process. This specific mutation was first described in a Sardinian patient with HbH disease [17] and since then has only been observed once, in a subject from Greece [30]. Consequently, our patient is the third case so far reported with the $\alpha\alpha/\alpha^{Nco}\alpha$ genotype.

The α-thalassemia defect is not uncommon in Spain but up to now only deletional forms of it have been described. These ten patients were the subset of nondeletional α-thal found in a larger group of 440 subjects (2%); the other 430 were carriers of different deletional forms of α-thal (mainly the $-\alpha^{3.7}$ deletion). Interestingly, the $\alpha\alpha/\alpha^{Hph}\alpha$ and $\alpha\alpha/\alpha^{Nco}\alpha$ genotypes identified here are the first description of nondeletional α-thal defects in Spain. Furthermore, since the deletion of 5 bp at the donor splice site of IVS I in the α_2-globin gene ($\alpha\alpha/\alpha^{Hph}\alpha$) was predominant (in 9 out of 10 cases), it may be suggested that this specific mutation is the most common nondeletional α-thal variant in this geographical area. It is worth mentioning, however, that none of these cases was a carrier of a poly A signal mutation (AATAAA→AATAAG) or a termination codon mutation of the α_2-globin gene, defects which are relatively frequent in other Mediterranean areas. The α_2-gene poly A signal mutation originally described in Saudi Arabia [31] has recently been found to be the most frequent nondeletional form of α-thal in Greece [30]. The same mutation has also been described, though less frequently, in other Mediterranean countries [32]. Likewise, the termination codon mutation in the α_2-globin gene was first described in a Greek individual [18] and has since only been reported in one Yugoslavian [33] and two Greek patients [30].

Therefore, our study demonstrates that these nondeletional forms of α-thal, frequent in some other Mediterranean countries are also present in Spain. Another point of interest is the absence of HbH in all the cases reported here. The mild hematological changes (microcytosis and hypochromia) found in these subjects in the absence of any major clinical abnormalities were consistent with the α-thal trait phenotype which, in our patients, results from the interaction of a normal haplotype (αα) with the α^+-thal defect produced by nondeletional mutations affecting the α_2-globin gene. Since these patients had a mild clinical phenotype, they were initially classified as carriers of α-thal on the basis of their hematological data, characterized by a decrease of the MCV associated with normal iron status and hemoglobin electrophoretic pattern.

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